

# Properties and Biological Significance of the Ileal Bile Salt Transport System

by Leon Lack\*

The properties of a specific transport system for bile salts, which is located in the ileum of the small intestine are described. The system operates by a sodium ion cotransport mechanism, and it functions in maintaining a normal enterohepatic circulation of bile salts. Analysis of structure-activity data allows us to depict our hypothesis for the interaction of the bile salt and Na with the membranal recognition site of this transport system. The sequelae of metabolic disorders which can arise following disease or surgical ablation of the ileal region of the intestine which result in an interrupted bile salt enterohepatic circulation are described. We suggest that these findings hold interest to toxicologists, since it is not beyond reason that toxic agents might exist which impair the function of this transport system specifically or which could poison the ileal mucosal cell. Such agents might be detected by the presence of some of the described metabolic disorders. Finally, we discuss the ileal transport of the sulfated esters of bile salts and the possibility that this might relate to that aspect of detoxification pertaining to their enhanced excretion.

Bile salts are detergentlike steroid derivatives which are synthesized in the liver from cholesterol. Their role in the digestive process has long been recognized. Bile salts are utilized within the intestinal lumen to emulsify lipid in order to facilitate its digestion. They function additionally by dispersing the products of lipid digestion, i.e., long-chain fatty acids and 2-monoglycerides, into micelles, thus allowing for their more efficient absorption. In the absence of bile salts the absorption of dietary lipid may be impaired by as much as 50%. The absorption of cholesterol and fat-soluble vitamins (A, E, and K) does not take place in the absence of bile salts. Bile salts are also required for the optimal function of pancreatic lipase. In addition, they have a critical role in maintaining cholesterol in a micellar state during the elaboration and storage of bile. It is believed that cholesterol gall stones can occur when a physiological imbalance between bile cholesterol and bile salts arises.

These various functions apparently require large quantities of bile salts. Hepatic secretion in man in the order of 30 g/day is about six times the total pool size and many times the rate of normal biosynthesis.

Enterohepatic circulation of the bile salts may be regarded as a mechanism for the conservation of these physiologically important substances. Figure 1 depicts this process schematically for man.

The pool size can be approximated as 3-5 g. In the normal preprandial state, the bulk of this material would be found in the gall bladder. Following the discharge of material from the gall bladder, the bile salts enter the intestine to become intimately associated with the foodstuffs in order for them to function in the manner already alluded to. Subsequent to the absorption of lipids from the proximal region of the small intestine, the bile salts are absorbed predominantly but not exclusively from the ileum. They then return to the liver by the portal route to be resecreted into the bile in order to continue the digestive absorptive process. Thus, during the digestion and absorption of a single meal this pool may circulate two or three times. The small amount of material escaping enterohepatic circulation (approximately 0.3 g/day) is restored by an equal conversion of cholesterol to bile salts by the liver. I would like to interject that this conversion in the liver represents a very real homeostatic process. Thus, if for some reason the enterohepatic process is interrupted for example, by a bile duct fistula or bile salt malabsorption, the liver increases the rate of conversion of cholesterol to bile salt by as much as sixfold in

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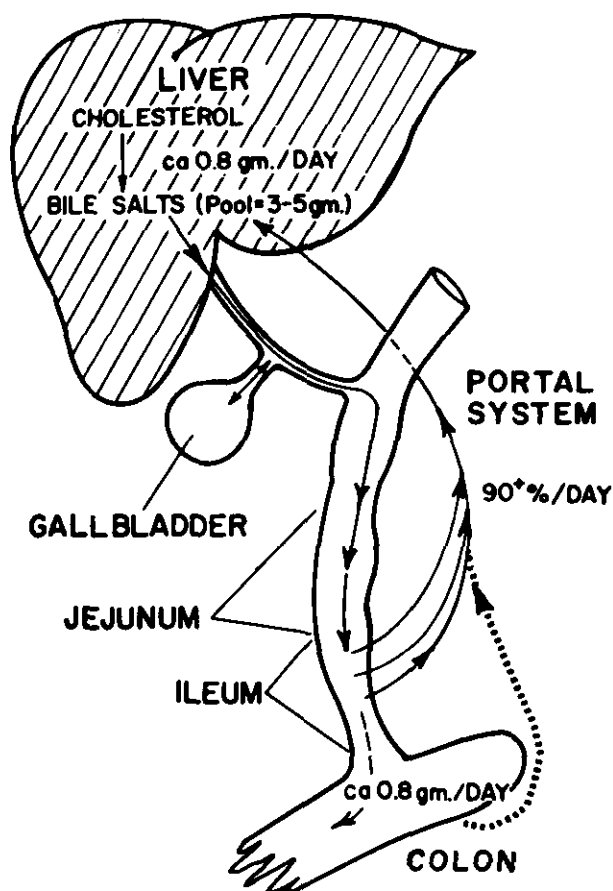


FIGURE 1. Schematic representation of the enterohepatic circulation of bile salts. The broken line represents bile salts which have been modified by bacterial action and reabsorbed from the colon by passive diffusion (28).

an attempt to maintain a normal bile salt pool. These aspects of bile salt physiology have been thoroughly reviewed (1-3).

As far as the intrainestinal events of lipid digestion are concerned, I would like to point out that this anatomical arrangement, where lipids are absorbed from the proximal region of the small bowel while the bile salts leave the intestine from the more distal region, could represent an adaptation that would allow optimal concentration of the detergent bile salts in the proximal region of the gut.

Some time ago we demonstrated that an active transport system for bile salt exists in the intestine and that this system exists only in the ileal region (4). These early *in vitro* observations were made with the everted gut sac preparation of Wilson and Wiseman (5). Some of these findings are shown in Figure 2.

The everted sacs were prepared from segments of the rat's small bowel. Each segment was one quarter

of the intestine. It can be seen that only those sacs prepared from the distal region can generate uphill movement from the mucosal to the serosal side of the gut wall, as evidenced by the final serosal to mucosal ratios greater than 1. This process is inhibited by sodium azide, dinitrophenol, and anoxia. Similar results were obtained when this type of experiment was performed with intestine sacs made from guinea pigs, hamsters, mice, spider monkeys, pigeons, and chickens (6).

Much of our *in vitro* work describing the nature of this process has been done with everted gut sacs prepared from guinea pigs. However, whenever possible, the specific *in vitro* observation in question was checked or documented with *in vivo* guinea pig models. The *in vivo* model involved perfusion of ileal or jejunal intestinal segments *in situ* in animals bearing common bile duct fistulae. It was possible to quantitate transmucosal movement of the test bile salts or derivatives by measuring the rate of their appearance in the secreted bile (7) which was collected via the cannula in the bile duct fistula (see Fig. 3).

Employing these models we have collected considerable data concerning structure-activity re-

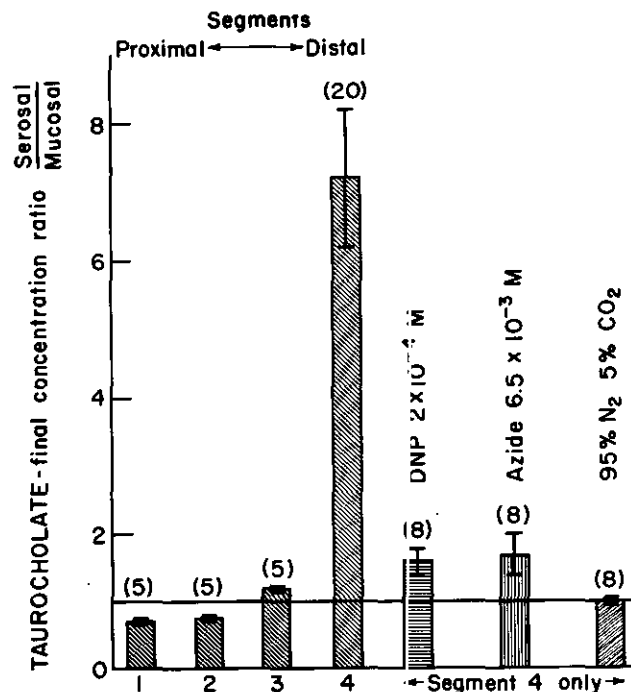


FIGURE 2. Transport of taurocholate by everted sacs of rat intestine. Each segment was one quarter the length of the small intestine. The numbers in parenthesis represent the number of animals in each group. The bars give  $\pm$  S.E. DNP = 2,4-dinitrophenol (4).

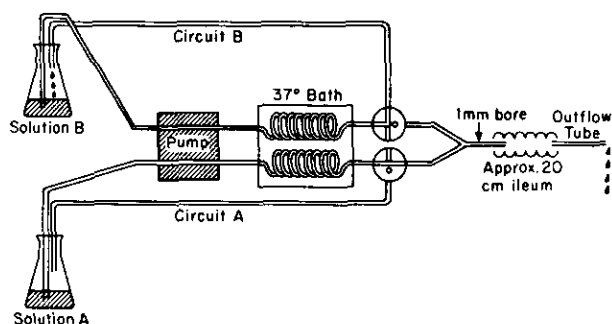
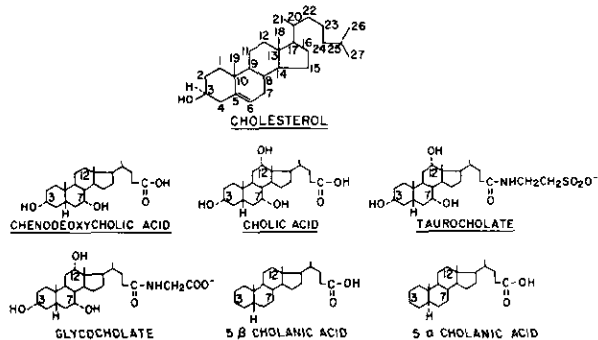


FIGURE 3. Illustration of the apparatus used for perfusing guinea pig ileum *in vivo* (4).

relationships and electrolyte requirements. Some of these findings will be reviewed in this communication. In addition, we will describe our hypothetical model for (bile salt) substrate — carrier interactions and then relate these findings and proposals to a discussion of those relevant aspects that might have potential interest to the toxicologist.



From the chemical structures of two important unconjugated bile acids — cholic acid and chenodeoxycholic acid — the chemical derivation from cholesterol is apparent. The biochemical pathways are rather complex and have been reviewed (1-3). Prior to being secreted into the bile these substances are conjugated with either taurine or glycine or a mixture of both. In man, the ratio of glycine to taurine conjugated bile salts is, under normal circumstances, about 3:1.

In our early structure activity studies we ascertained that no particular hydroxyl group on the bile salt was essential for its active transport (Fig. 4). The three derivatives of taurocholanolic acid — 3,12-dihydroxy-, 3,7-dihydroxy-, and 7,12-dihydroxy — can all be transported by everted gut sacs from the ileum. In essence, we have taken taurocholate — the 3, 7, 12-trihydroxy compound whose transport was already demonstrated (4) — and selectively removed a specific hydroxyl group from each position

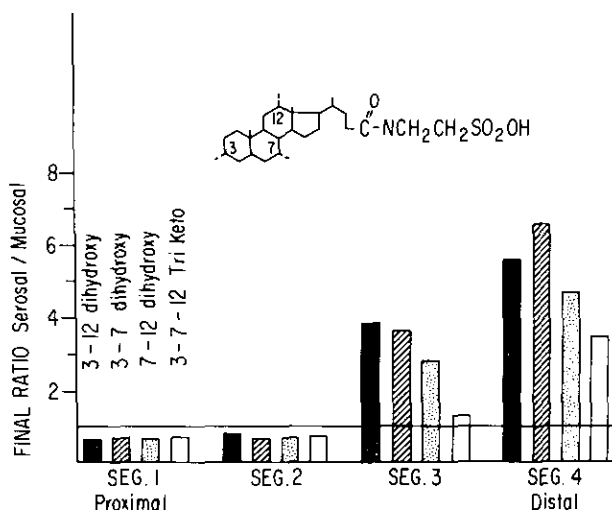


FIGURE 4. Transport of various bile salts by everted sacs of guinea pig small intestine. Figure is a composite of previous data (8).

and still maintained transport activity. The triketo compound is not a natural substance. Here one can envision taurocholate with its three hydroxyl groups oxidized to three keto groups. This compound completely devoid of hydroxyl substituents still retains some residual capacity to act as a substrate for this transport system. It should be noted that the formation of the triketo compound results in the stereochemical distortion of the normal shape of the cholanolic acid steroid.

When we studied the mutual inhibition between the di- and trihydroxy compounds we ascertained on the basis of *in vivo* and *in vitro* studies that (1) dihydroxy bile salts are better inhibitors than the trihydroxylated compounds; (2) trihydroxylated compounds are more readily inhibited than the dihydroxylated substances; (3) the triketo compounds (which I have mentioned has a distorted steroid structure) are the poorest inhibitors and the compound most readily inhibited (7, 8).

Thus, while the transport system does not absolutely require a specific hydroxyl group for interaction, the hydroxyl groups do influence transport activity, in that there appears to be an inverse relationship between the number of hydroxyl groups on the steroid nucleus and the apparent affinity between substrate and transport system as indicated by the mutual inhibition studies. This seeming anomaly remains to be explained and would have to be considered in any hypothesis concerning substrate-carrier interaction.

At physiological pH the natural bile salts all possess a single negative charge on the side chain. Our structure activity studies would appear to indicate

that this structural element — a single negative charge on the side chain — was essential for optimal transport. Figure 5A compares the *in vitro* transport of taurocholate with two dibasic or dianionic derivatives. It can be seen that the dibasic substances were poorly transported when compared with their natural analog. Figure 5B compares the effects of altering the pH of the incubation media on this process. Glycocholate is the natural analog, and the carboxymethyl derivative cholyaspartate is the test substance. At lower pH the transport of glycocholate decreases, while that of cholyaspartate increases in absolute terms and relative to that of glycocholate. Since one would expect that more of the singly charged species of cholyaspartate would exist at lower pH, its enhanced uphill movement with lower pH would agree with the concept that a single negative charge on the side chain is a critical structural factor. These findings were confirmed with similar studies in *in vivo* preparations (10).

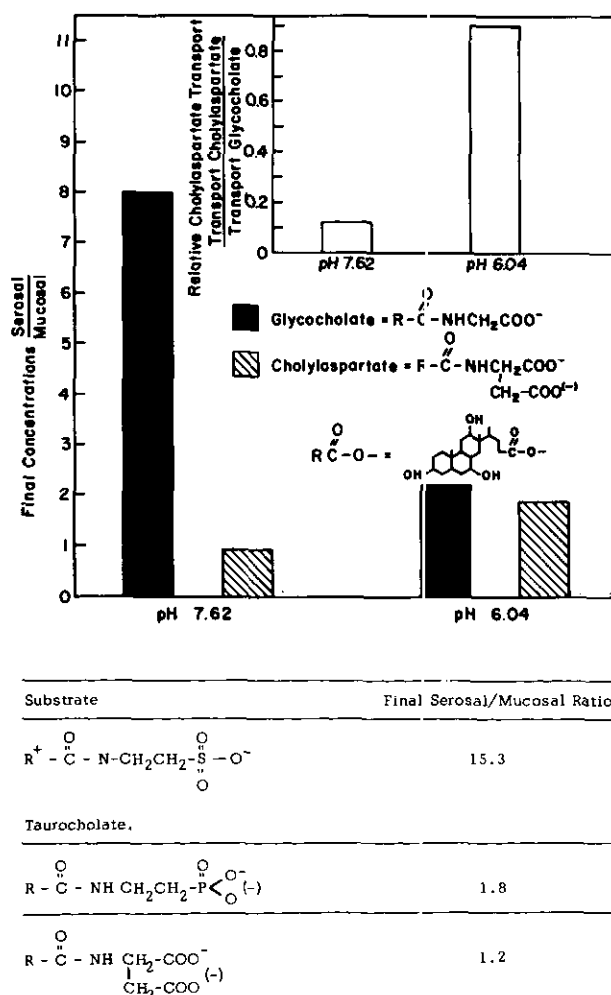
It may be of interest to consider reasons why a substrate bearing two negative charges in this region of the molecule should not be transported. A possible explanation would be that during normal transport the bile salt interacts with an active site bearing a positive charge. If a negative charge existed in the region of this active site, sufficient repulsion might exist between the transport system and the second negative charge on the unnatural substrate to prevent transport. One may speculate further concerning the nature of this proposed negative charge in the region of the active site. If under normal conditions it reacted with sodium, it might be functional in the transport process, since sodium ions are necessary for the transport of bile salts (11, 12).

To accommodate the above speculation we propose that the initial interaction of the bile salt substrate and sodium with the membrane recognition site or carrier occurs in a manner depicted in Figure 6.

Three components of interaction are involved: (a) an interaction of the steroid part of the bile salt and the carrier; (b) a coulombic interaction between the negatively charged side chain and a positively

**Table 1. Metabolic disorders resulting from ileal disease.**

Steatorrhea (>5.5 g/24 hr with intake of 70-90 g LCT/24 hr)
Watery diarrhea (>200 g/24 hr, Na > 7 meq/24 hr, K > 20 meq/24 hr)
Hypercholesterolemia or lower range of normal
Relative deficiency of taurine (glycine: taurine of conjugated bile salts considerably > 3:1)
Bile supersaturated with cholesterol (gallstones)
Oxaluria (nephrolithiasis)
B <sub>12</sub> deficiency (abnormal 2nd stage Schilling test)



**FIGURE 5.** Effect of pH on transport of glycocholate and cholyaspartate (9) and comparison of taurocholate transport with dibasic analogs.

charged element in the membrane or carrier (designated cationic site), and (c) a closely positioned function of negative charge on the carrier which can interact with the sodium ions. Furthermore, we propose that these three components of interaction function cooperatively, and that this structure or recognition site resides within a hydrophobic crevice or pouch in the membrane. This complex of speculations could explain the results of our mutual inhibition studies. Thus, even though no specific hydroxyl group is required for a critical interaction between carrier and substrate, those compounds with fewer hydroxyl groups would have better accessibility to the hydrophobic space; in mutual inhibition studies, for example, between di- and trihydroxylated substrates, more of the dihydroxy compound would

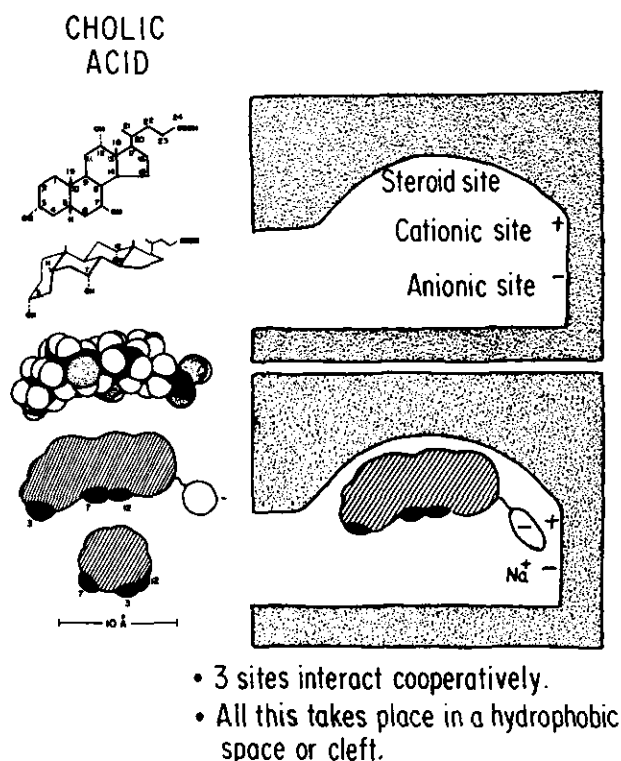


FIGURE 6. Hypothetical scheme for the interaction of a bile salt with its recognition site in the membrane. Also shown are the various ways of depicting the cholic acid moiety. The representational structures of cholic acid shown on left were taken from the literature (1).

partition into this region and perform as a better inhibitor. However, the oil/water partition solubilities of the triketo compounds lie between those for their trihydroxy and dihydroxy analogs. Yet they are the weakest inhibitors and the compounds most readily inhibited. We have attributed this to the fact that the already stated coplanarity requirements of the three keto groups have distorted the regular cholestane configuration common to the natural bile salts, and one of the components of interaction namely that between the membrane carrier and the steroid moiety is not optimal.

Such a complex hypothesis would dictate predictions which are testable. For example, these speculates would predict that the Na requirements for transport of the triketo bile salt substrate be greater than that for its natural analog. That this is so is shown in Figure 7. These are *in vitro* transport studies. The incubations involved the usual Krebs-Ringer buffers with varying amounts of NaCl replaced by isoosmotic equivalents of mannitol. As we decrease the concentration of Na in the incubations involving taurocholate, there is a significant but not too dramatic decrease in transport. Note that at 30

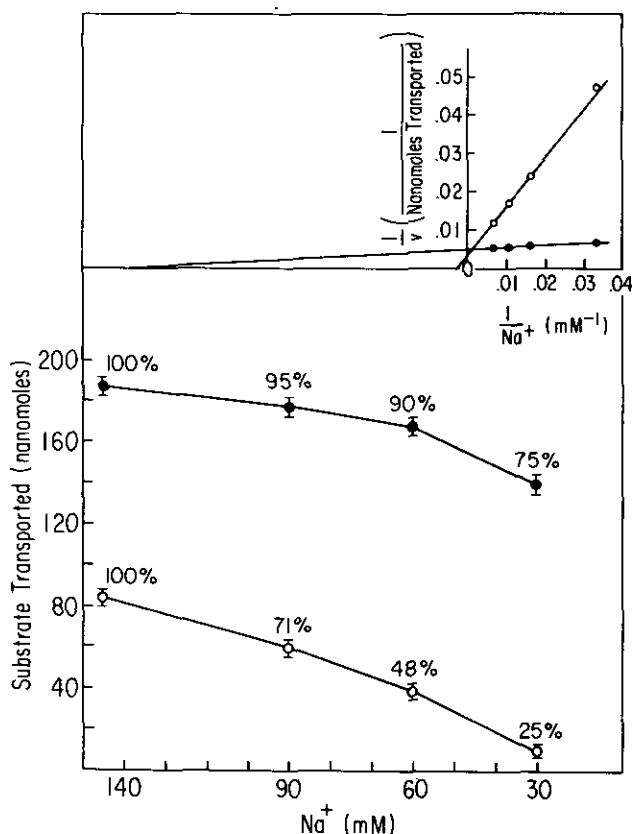


FIGURE 7. Comparison of the *in vitro* transport of taurocholate with taurodehydrocholate (12) in media of different Na<sup>+</sup> concentrations. (●) transport of taurocholate (each point is the mean  $\pm$  SEM of eight gut sacs); (○) transport of taurodehydrocholate (each point is the mean  $\pm$  SEM of 16 gut sacs. Solutions were Krebs-Ringer bicarbonate with different amounts of Na<sup>+</sup>. Mannitol was used as the osmotic replacement for NaCl. Concentration of substrate 28 nmole/ml.

mM Na<sup>+</sup> transport is still 75% of control values. However at comparable Na levels, transport of the distorted triketo analog of taurocholate is inhibited by 75%. Also shown are the double reciprocal plots of this data. Reference to the intercepts of the abscissa would show that the apparent affinity for Na by the transport system is much greater in the presence of taurocholate than in the presence of the triketo analog.

The proposition of cooperativity would require that mutual inhibition studies between taurodehydrocholate and taurocholate demonstrate that taurodehydrocholate would function as a better inhibitor at higher Na ion concentration than at lower Na levels. As one lowers the Na concentration, the interaction of the distorted triketo compound with the transport system would decrease more than that of the natural compound and therefore act as a less capable inhibitor. This was found to be the case (12).

The hypothetical scheme would predict that bile salts modified in a manner such that there be no charge on the side chain would still be capable of interacting with the transport site by virtue of the steroid recognition component but that uphill transport should be depressed dramatically because the coulombic interaction and the cationic membrane site could not take place. When such compounds were synthesized and tested (13), we were able to demonstrate interaction as evidenced by the fact that *in vivo* studies demonstrated preferential ileal absorption. In addition, these compounds could inhibit *in vitro* bile salt transport in a manner that would be expected from our earlier mutual inhibition studies with the natural bile salts; i.e., the fewer the hydroxyl groups, the better these compounds function as inhibitors. In addition, the triketo analog is without effect. Uphill transport (against a concentration gradient) by everted gut sacs is either minimal or not observed, depending on the analog tested. Figure 8 demonstrates this transport with our most active analog, cholyl NPG. It is apparent that this observed transport is much less than that shown by the natural congener, taurocholate. The proposal for cooperativity between the various sites would predict that the transport of cholyl NPG would be more sensitive to Na ion depletion than its anionic analog. This too, has been demonstrated.

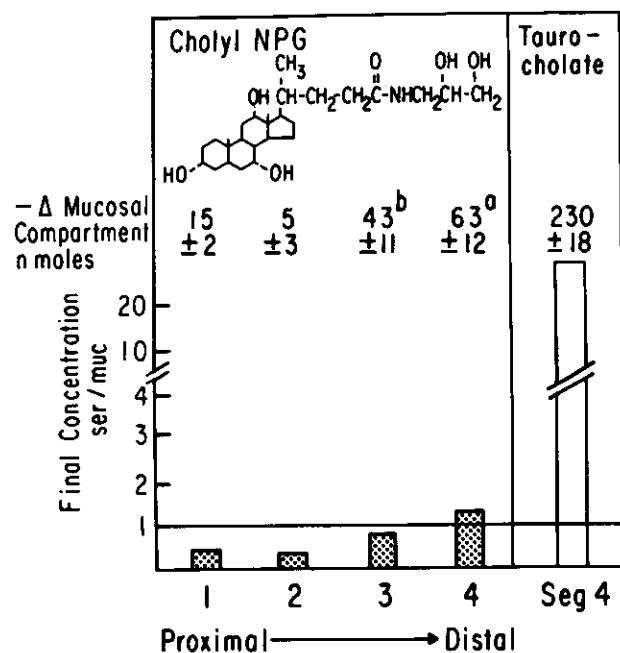


FIGURE 8. Transport of an uncharged bile salt derivative by everted gut sacs prepared from different regions of the guinea pig small bowel. Also shown is the transport of taurocholate in parallel incubation of gut sacs from distal small bowel. (13).

If the proposed scheme for interaction were correct, one would expect that positively charged or cationic analogs might not be transported at all. If the steroid moiety of the derivative could still interact at the steroid recognition site, then such compounds would act as refractory substrates, and inhibition of the transport of natural bile salts could take place. Furthermore, the proposal would insist that the order of inhibition of bile salt transport by the positively charged derivatives follow the same order observed in the mutual inhibition studies, i.e., the cationic derivative with one hydroxyl group would be a better inhibitor than those derivatives with two hydroxyl groups. The trihydroxy derivative would be even less effective as an inhibitor and, of course, the triketo compounds even less potent. This order of inhibition was observed in *in vitro* and *in vivo* studies (14).

The proposal would suggest that the cotransport of the Na cation and the bile salt anion from the lumen of the intestine across the ileal brush border membrane into the mucosal cell might be an electroneutral process. In other words, since the loaded carrier is depicted as neutral, transmembrane movement of the bile salt could be to a great extent, if not completely independent of the nature of the anion in the incubation media. This would be in contrast to the known transport processes involving glucose (15).

Vesicles were prepared from intestinal brush border membranes obtained from guinea pig ileum and jejunum. It was possible to demonstrate enhanced taurocholate uptake by vesicles made from ileal tissue which was dependent on the presence of a gradient of Na ions. Vesicles made from jejunal tissue did not demonstrate such activity. These preliminary data are demonstrated in Figure 9. Proximal or distal vesicles were incubated with mannitol and <sup>14</sup>C-taurocholate. At the point indicated by the arrow, solutions of NaCl (Fig. 9A) were added containing the <sup>14</sup>C-taurocholate at the same concentration as that in the incubation media. Note that there is an overshoot of Na taurocholate uptake with ileal vesicles which presumably can be maintained until the Na activity inside the vesicles equals that on the outside. With proximal vesicles the increase in uptake is modest, presumably reflecting the swelling of vesicles following the diffusion of electrolyte. The data in Figures 9E and 9F demonstrate that neither KCl or LiCl can replace NaCl. However, NaCNS, Na isethionate, or Na<sub>2</sub>SO<sub>4</sub> can effectively replace NaCl. Thus, the magnitude of the overshoot phenomenon is not altered when the chloride ion is replaced by a more permeant anion (Fig. 9B) or less permeant anions (Figs. 9C, 9D).

Finally, this model might have something to say

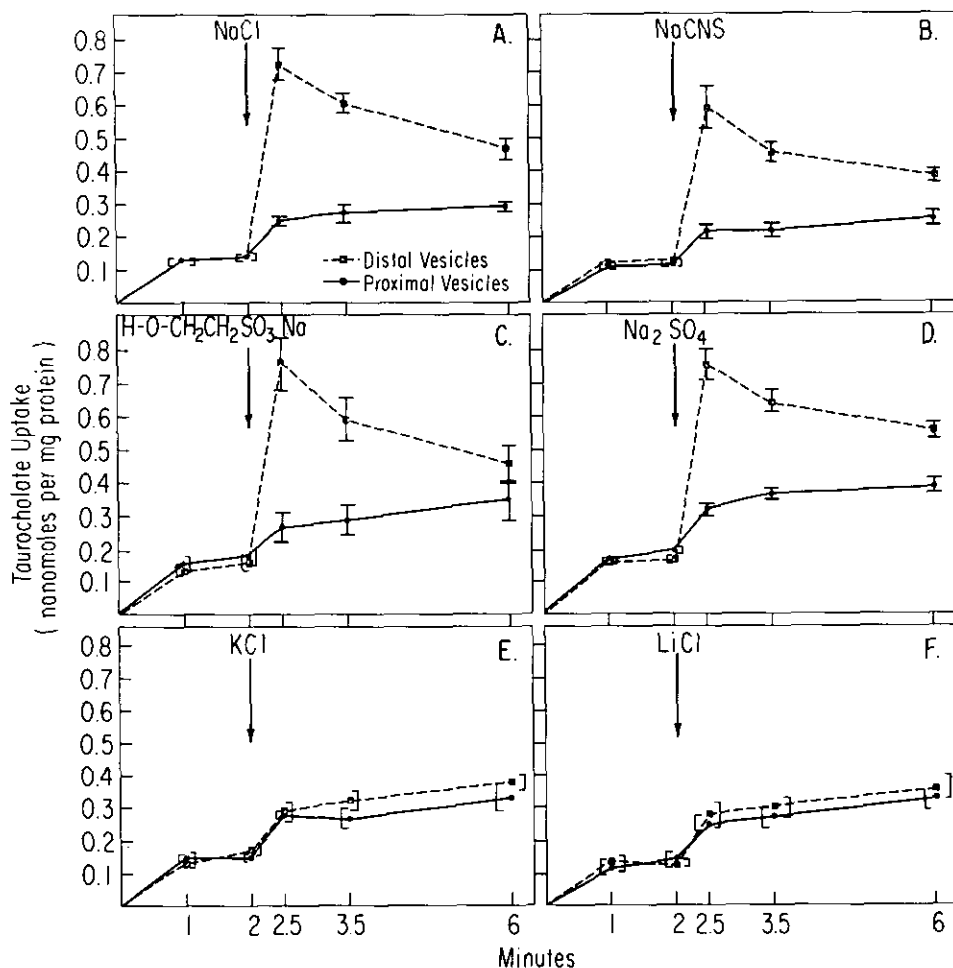


FIGURE 9. Uptake of 24-[<sup>14</sup>C]-taurocholate by brush border membrane vesicles in the presence of experimental electrolytes. Buffered electrolyte solutions were added at the times indicated. Each value represents the coverage of four to eight experiments. Also shown are the standard errors of the means (16).

concerning the position of the second negative charge which, as we mentioned, appears to abort transport when introduced on the side chain. Thus, as one moves the second potential negative group away from the side chain area where the coulombic interactions are presumed to occur, the question arises whether substrate-carrier interaction could more likely occur. We will have something to say about this when we discuss the effects of bile salt sulfation and its relevance to toxicity. Let us proceed to discuss the physiological implications of this transport system which can move bile salts out of the lumen into the portal circulation and which is present only in the ileum. There is no doubt that absorption of bile salts can, to some degree, take place by passive fluxes along the entire length of the small and large intestine. We will not discuss the discussions that are current concerning the quantitative role that

these processes contribute to the overall enterohepatic circulation. Certainly these (passive) processes are real, and furthermore, the tendency for such diffusion increases as the number of hydroxyl groups decreases. We will see that this becomes a very real problem when we discuss the enterohepatic circulation of the monohydroxy bile salts of lithocholic acid, a secondary bile salt with toxicological implications. However, as far as the ileal transport is concerned, it is generally accepted that the removal of the ileum effectively interrupts the enterohepatic circulation of bile salts. This is in contrast to the removal of proximal regions of the intestine, where it can be demonstrated that the biological half-life of the bile salt pool is minimally affected.

With the loss of bile salt enterohepatic circulation following ileal resection, the hepatic feedback mechanism makes an attempt at compensation by en-

hancing the daily conversion of cholesterol to bile salts by several fold. In this manner, enhanced amounts of bile salts now enter the colon. This was first demonstrated by Weiner, Playoust, and Lack with surgically prepared dogs at Johns Hopkins (17). Following my arrival at Duke, I had the opportunity to collaborate with Dr. Tyor in the G.I. Division of the Department of Medicine and we found that the same pertained to patients with ileal disease or with patients with ileal resection performed as consequence to ileal disease (18, 19).

Surgical removal of the ileum for certain disease states is widely practiced. Certain sequelae have been recognized to occur as a consequence. Some metabolic disorders resulting from lack of ileum function are steatorrhea ( $> 5.5$  g/24 hr with intake of 70-90 g LCT/24 hr); watery diarrhea ( $> 200$  g/24 hr,  $\text{Na} > 7$  meg/24 hr,  $\text{K} > 20$  meg/24 hr); hypocholesterolemia or lower range of normal; relative deficiency of taurine (glycine: taurine of conjugated bile salts considerably less than 3:1); bile supersaturated with cholesterol (gallstones); Oxaluria (nephrolithiasis); and  $\text{B}_{12}$  deficiency (abnormal 2nd stage Schilling test).

Steatorrhea or impaired lipid absorption stems from the fact that in the absence of ileal function recirculation of bile salts during the course of a day is interrupted. In spite of enhanced biosynthesis of bile salts, after the first meal following the overnight fast, adequate hepatic bile salt secretion cannot be maintained.

Without a functioning ileal bile salt transport system the amount of bile salts daily entering the colon is increased. Enteric bacteria have the property of deconjugating the bile salts and modifying the steroid structure forming secondary bile salts. An important one is deoxycholic acid derived from the 7-dehydroxylation of cholic acid. The increased levels of unconjugated dihydroxy bile acids have the property of inhibiting the Na K ATPase of the colon. With the consequent decreased levels of electrolytes and water absorption one very frequently sees a watery diarrhea (20, 21).

Hypocholesterolemia would appear to be due to the increased drainage on the cholesterol pool following the enhanced conversion of cholesterol to bile salts.

Man is a species whose bile salt pool consists of glycine and taurine conjugates in an approximate ratio of 3: 1. Taurine is biosynthesized by the liver from cysteine. With enhanced biosynthesis following the loss of ileal function greater proportions of bile salts are elaborated as glycine conjugates. This enhanced glycine to taurine ratio is primarily of hepatic origin, probably reflecting inadequate avail-

ability of hepatic taurine from cysteine (22, 23).

In the absence of adequate amounts of bile salts in the elaborated bile one obtains a biological imbalance with a tendency for the cholesterol to precipitate out. Following his two-year visit to our laboratory, Dr. Ken Heaton returned to England and did a retrospective study and ascertained that patients who had their ileums removed had a higher than normal incidence of gallstones (24). These enhanced statistics became more apparent with greater time lapse; following resection the incidence of gallstones increased.

With ileal disease an increase of renal stones occurs. These stones more often than not are of the calcium oxalate type. Upon removal of the ileum one can obtain hyperoxaluria (25).

Two factors are probably involved here: with the steatorrhea following loss of ileal function, increased amounts of fatty acids remain in the intestine and are excreted in the feces. Fatty acids have a tendency to react with  $\text{Ca}^{2+}$  in the intestinal contents to form insoluble Ca soaps. It would appear that  $\text{Ca}^{2+}$  normally reacts with dietary oxalate as a means of inhibiting oxalate absorption. The decreased availability of free  $\text{Ca}^{2+}$  in the intestine following the formation of Ca soaps could result in enhanced oxalate absorption. In addition, it has been shown that the excess secondary bile salts affect the permeability of the large bowel to oxalate ions, and this too could be a contributing factor (26).

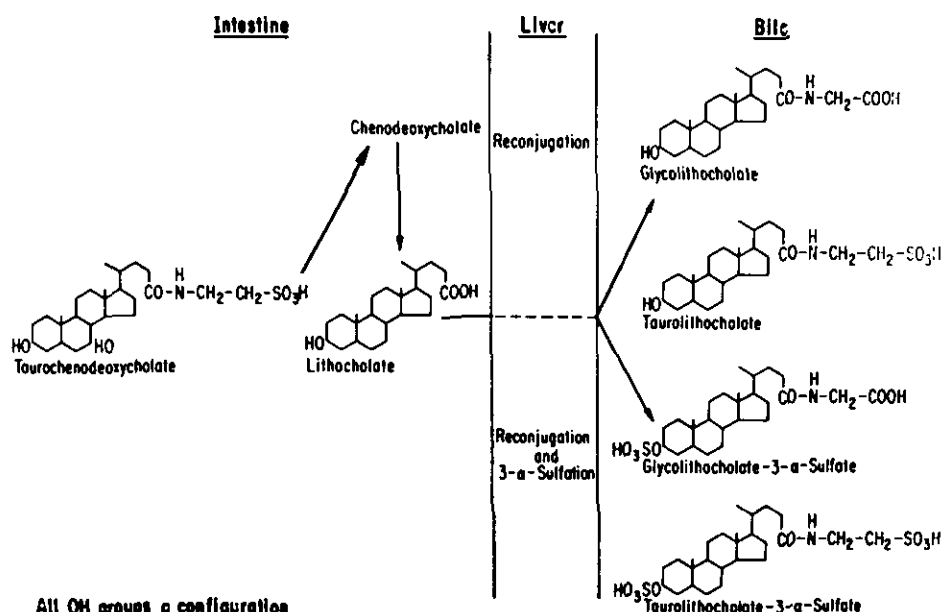
The  $\text{B}_{12}$  deficiency is not related to the loss of the bile salt transport system but to the fact that the specialized system for absorbing the  $\text{B}_{12}$  intrinsic factor complex also exists exclusively in the ileum.

Recently two cases were reported by Heubi et al. (27) of children showing some of these disorders — and this led the investigators to suspect that these children had a genetic deficiency of the ileal bile salt transport system. Their uptake studies with biopsy tissue would appear to indicate that this deficiency does indeed exist. Interestingly enough, they report that  $\text{B}_{12}$  absorption was normal.

Metabolic disorders listed above should be of potential interest to the toxicologist. We suggest that should an incidence of intoxication include any of these disorders one ought to think in terms of ileal function.

I have already alluded to the phenomenon that bile acids can be modified by intestinal bacteria to form a group of substances referred to as secondary bile salts. Lithocholic acid is a monohydroxylated bile acid that is formed in the intestine from conjugated chenodeoxycholate compounds as a consequence of bacterial removal of the glycine or taurine moiety and the OH group in the 7- $\alpha$  position (Fig. 10). It can





All OH groups α configuration

FIGURE 10. Metabolic transformation of glycocholic acid during enterohepatic circulation. A similar sequence is operative for taurocholic acid. Glycine and taurine conjugates of chenodeoxycholic acid, which have OH groups at the 3-α and 7-α-positions also undergo deconjugation and 7-α-dehydroxylation (28).

then return to the liver for further processing. Lithocholate acid and its taurine and glycine conjugates have been implicated in a variety of toxicological processes which were observed by several investigators in a number of experimental animal models. These include cirrhosis of the liver, bile duct hyperplasia, and gallstone formation. Following intramuscular injection in man, such lithocholates have been reported to cause local inflammation, malaise, and fever. This subject has been reviewed by Palmer (29).

In 1967 Palmer reported that the liver was capable of sulfating lithocholate bile salts (30, 31). This was a very important observation, since it represented the description of a new metabolic pathway for bile salts (Fig. 10). Implications pertaining to detoxification became immediately apparent. Lithocholate, being a monohydroxylated bile salt, is less water-soluble and more lipid-soluble than substances with two or three hydroxyl groups. As a result, one observes significant passive diffusion across the intestine. Figure 11 shows results of the *in vivo* intestinal absorption studies of these compounds, done with guinea pigs (32). Distal and proximal segments were perfused and transmucosal absorption was monitored by following the recovery of the test substrate in the bile. On the left of Figure 11 we see that considerable absorption of taurolithocholate and glycolithocholate can be observed from proximal as well as distal regions, suggesting that both passive

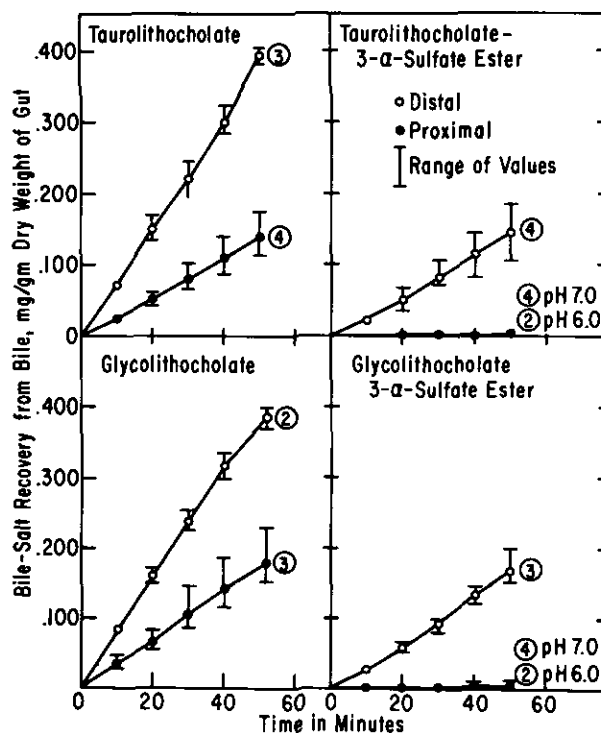


FIGURE 11. Absorption of taurolithocholate and glycolithocholate (left) and of their 3-α-sulfate esters (right) as measured by recovery of radioactivity from a bile fistula after *in vivo* perfusion of proximal and distal segments of small intestine from (32).

process and active transport systems can operate in absorbing these substances. Following sulfation, one can detect absorption only from the distal region of the small bowel suggesting that the passive flux of these three sulfate derivatives is minimal. Furthermore, ileal absorption of the sulfated compound could be inhibited when they were perfused together with primary bile salts. These animal studies suggested that the hepatic sulfation in the case of the lithocholates might be an adaptive mechanism to enhance their fecal excretion. Subsequently, Hofmann et al. observed essentially the same pattern in human studies (33).

It must be noted that these sulfate esters bear two negative charges at physiological pH. We have stated earlier that much of our ideas concerning carrier-substrate interactions rests on the observation that bile salts modified to have two negative charges on the side chain appeared not to be transported. Here when the second negative charge was introduced at the other end of the molecule there was obviously some transport. *In vitro* studies confirm these *in vivo* observations. Although the 3-position is displaced from the side-chain region, we were still not completely comfortable with our conclusions concerning two charges on the molecule. Therefore, it was important to ascertain whether sulfate esterification had a quantitative effect on substrate transport. Unfortunately, the properties of tauro-lithocholate and glycolithocholate were such as to prevent a quantitative evaluation of the effect of sulfation at this position. As demonstrated, conjugated lithocholates being monohydroxylated bile salts can to a great extent cross the intestinal mucosa

by passive means. In addition, these substances have a strong tendency to bind to tissue in a non-specific manner. Therefore, *in vivo* and *in vitro* evaluations of the ileal transport of lithocholic acid conjugates could not be accurately assessed. These critical complications of passive fluxes and non-specific tissue binding do not pertain to the naturally occurring di- and trihydroxylated bile salts. Therefore, when it was reported that sulfation of di- and trihydroxylated bile salts can take place, and that these processes are enhanced in patients with hepatobiliary disease (34-37), we decided to reinvestigate this question. The primary bile salt, taurochenodeoxycholate, was selected because it allows for the assessment of the effect of sulfation at the 3-position, the 7-position, and the 3,7-position. These bile salt esters were prepared and tested for their ability to be absorbed by the intestine (38). *In vivo* perfusion of segments of small bowel with labeled sulfate esters showed that sulfation markedly decreased transport by the ileal bile salt transport system and that the position and number of the sulfate radicals was directly correlated with the degree of transport inhibition. The following structure relationships were found: transport of taurochenodeoxycholate (TCDC) > TCDC-3-sulfate > TCDC-3,7-disulfate with a decrease in magnitude of approximately 90% between each pair (Table 2). Sulfation thus can be envisioned as a means of enhancing excretion by the fecal route in conditions of partial obstruction. It would also appear that ileal transport of the monosulfates is less when the sulfate ester is closer to the side chain.

Enhanced renal excretion of sulfated bile salts has

**Table 2. Intestinal absorption of sulfated derivatives of taurochenodeoxycholate from distal and proximal regions of the small bowel of guinea pigs.**

Substrate	Proximal segments		Distal segments	
	No. of animals	Absorption, nmole/g dry wt.	No. of animals	Absorption, nmole/g dry wt.
Taurochenodeoxycholate	4	73.2 ± 7.2	4	2290.0 ± 160.2 <sup>a</sup>
Taurochenodeoxycholate-3-sulfate	4	4.7 ± 1.7	5	203.0 ± 24 <sup>b</sup>
Taurochenodeoxycholate-7-sulfate	5	3.1 ± 0.8	5	25.6 ± 4.2 <sup>c</sup>
Taurochenodeoxycholate-3,7-disulfate	4	4.8 ± 1.8	4	3.4 ± 0.9 <sup>d</sup>

<sup>a,b,c,d</sup>These values for ileal absorption are significantly different from each other: a and b,  $p < 0.0001$ ; b and c,  $p < 0.0001$ ; c and d,  $p < 0.001$  by Student's *t*-test. In the case of the experiments with taurochenodeoxycholate (TCDC), TCDC-3-sulfate, and TCDC-7-sulfate, distal absorption was significantly greater than the respective proximal absorption:  $p < 0.001$  in all cases. Distal and proximal absorption of the TCDC-3,7-disulfate were not significantly different from each other.

Perfusion solutions consisted of normal saline buffered with 0.01 M Na phosphate, pH 6.9 with bile salts added in a concentration of 32 nmole/ml. The rate of perfusion was 3.5 ml/min. Perfusion with labeled bile salt was performed for a standard period of 60 min. This was preceded by a flush with buffered normal saline for at least 10 min and was followed by perfusion with buffered normal saline until no further radioactivity appeared in the bile (38).

been demonstrated by patients with biliary obstruction. Its relevance to the problems under consideration stems in part from an early observation of ours that a similar transport system (for bile salts) exists in the renal tubule which operates in the reabsorptive direction (39). If it has the same structure-activity characteristics as the ileal transport system, then the bile salts following sulfation would not be reabsorbed as readily as their unsulfated progenitors and as such be more likely to be excreted in the urine.

The author's work reported here was supported by Grant AM09582 from the National Institute of Health.

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